Pre-natal stress and post-natal pain affect testis development in young pigs.

REPRODUCTION
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Pre-natal social stress and post-natal pain affect the developing pig reproductive axis

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Abstract

This study assessed the effect of pre-natal social stress and post-natal pain on the reproductive development of young (approximately day 40) pigs. Male pigs carried by sows that were stressed by mixing with unfamiliar older sows for two 1-week periods during mid-pregnancy had lower plasma testosterone (0.54 vs 0.86 ng/ml, S.E.D.=0.11; $P=0.014$) and oestradiol (E_2; 22.9 vs 38.7 pg/ml, S.E.D.=7.80; $P=0.021$) concentrations compared with males carried by unstressed control sows. Although there was no effect of pre-natal stress on female E_2 concentrations, female pigs carried by stressed sows had fewer primordial ovarian follicles (log $-4.32/\mu m^2$ vs $-4.00/\mu m^2$, S.E.D.=0.136; $P=0.027$). Tail amputation on day 3 after birth reduced E_2 concentrations in female (4.78 vs 6.84 pg/ml, S.E.D.=0.86; $P=0.03$) and in male (25.6 vs 34.9 pg/ml, S.E.D.=3.56; $P=0.021$) pigs and reduced both testis weight (0.09% of body weight vs 0.10% of body weight, S.E.D.=0.003; $P=0.01$) and the percentage of proliferating Leydig cells (1.97 vs 2.12, S.E.D.=0.114; $P=0.036$) compared with sham-amputated littermate controls. There was a significant ($P=0.036$) interaction between the effects of pre-natal stress and post-natal pain on testicular expression of the steroidogenic enzyme 17α-hydroxylase, such that amputation increased expression in pigs born to control sows, but reduced expression in animals born to stressed sows. This study shows that stressful procedures associated with routine animal husbandry can disrupt the developing reproductive axis.

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Introduction

It is widely recognised that the developing foetus and neonate is sensitive to changes in the external maternal environment, resulting in alterations in a range of physiological systems, including reproductive processes, in the foetus, neonate and resultant adult offspring (Ashworth *et al*. 2005). In livestock species, the majority of studies investigating the effects of pre- and early post-natal conditions on reproductive traits have focused on effects of altered maternal nutrition (typically under nutrition) during pregnancy and lactation. In sheep, for example, under nutrition during different stages of gestation reduced folliculogenesis in foetal ovaries (reviewed by Rhind (2004)), ovulation rates at first oestrus (Rae *et al*. 2002) and the number of Sertoli cells in the testes of male lambs (Bielli *et al*. 2002). A recent review highlighted that early post-natal life experiences are also important for lifetime fertility (Gardner *et al*. 2008). Less is known about the effects of other, non-nutritional, changes to the maternal environment on the developmental programming of the reproductive axis in livestock species.

Evidence from several mammalian species suggests that various forms of pre-natal stress (including social, spatial and environmental) affect the reproductive competence of offspring. For example, social stress evoked by short, frequent meetings of pregnant bank voles reduced the rate of offspring sexual maturation, uterine and testis weights and altered the attractiveness of adult offspring to the opposite sex (Marchlewska-Koj *et al*. 2003). Studies in which pregnant rats were stressed by several periods of restraint under intense light during the last week of pregnancy (Ward & Weisz 1984) concluded, based on foetal plasma testosterone and progesterone concentrations, that the male foetal reproductive axis was more susceptible to pre-natal stress. Following this, many studies measured male offspring testosterone concentrations or the anogenital distance, which reflects the migration of the genital orifice towards the navel during foetal life and is often used as an indicator of masculinisation, as markers of pre-natal stress. Male guinea pigs born to mothers stressed by exposure to a strobe light during late pregnancy (Kapoor & Matthews 2005) and male rats born to dams mixed with lactating rats during late

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pregnancy (P J Brunton, M V Donadio, S T Yao, M Greenwood, D Murphy & J A Russell 2010, unpublished data) had lower plasma testosterone concentrations. However, Kattesh *et al.* (1979) reported no effects of stressing pregnant sows with heat and crowding for 50 days during mid-pregnancy on testosterone concentrations or libido of male offspring. Shorter anogenital distances in male offspring have been observed in prenatally stressed male rats (Williams *et al.* 1998, Mairesse *et al.* 2007) and in male pigs born to sows receiving weekly injections of ACTH during mid-pregnancy (Lay *et al.* 2008).

Many experimental situations used to create pre-natal stress do not reflect typical conditions that could be experienced by pregnant females. In pigs, EU legislation recommends that pregnant sows and gilts are housed in groups throughout pregnancy to ensure that their movement is not restricted and that animals can socially interact. However, as pigs form social dominance relationships, group housing with unfamiliar sows can be associated with aggressive interactions and social stress in subordinate animals (Jarvis *et al.* 2006). In addition, piglet's tails are often removed within a few days of life as a means to prevent later tail biting; a procedure which causes an acute stress response (Sutherland *et al.* 2008) and behaviour indicative of pain perception (Rutherford *et al.* 2009). Although there are apparently no reports of the effects of early post-natal pain or trauma on subsequent reproductive function, it is known that male pigs that were handled daily for the first 2 weeks of life had altered hypothalamic–pituitary–adrenal (HPA) function when assessed at 7 months of age (Weaver *et al.* 2000).

Our preliminary studies (Harker *et al.* 2008) which surveyed changes in a range of steroidogenic and gametogenic factors in pig ovaries and testes including aromatase, 3β-hydroxysteroid dehydrogenase, androgen receptor, LH receptor, STAR protein and VASA, indicated that testicular expression of the steroidogenic enzyme 17α-hydroxylase (17α-OH), which is involved in sexual development during foetal life, was affected by both pre-natal stress and post-natal pain. The objectives of this study were to determine if the stress associated with routine animal husbandry practices could affect the normal pattern of development of the reproductive axis and whether gender differences in the sensitivity to such stressors exist.

Table 1 Effect of mid-gestation stress on gestation length, litter size and piglet weight.

	Control	Stressed	Effect of pre-natal treatment
Gestation length (days)	115.3±0.59	114.1±0.30	NS
Total litter size	12.8±1.03	13.4±0.53	NS
Number born alive	12.8±1.00	12.4±0.80	NS
Total litter weight (kg)	16.0±1.08	16.9±1.33	NS
Average litter weight (kg)	1.30±0.0498	1.34±0.0834	NS
Within-litter standard error of birth weight	0.05221±0.00654	0.05359±0.00449	NS

Values are mean±S.E.M. NS, not significant.

Table 2 Weight (kg) of the 64 pigs studied at birth and at slaughter.

		Control		Stressed	
		Intact	Amputated	Intact	Amputated
Birth	Female	1.35±0.065	1.34±0.078	1.35±0.10	1.37±0.10
	Male	1.34±0.036	1.34±0.060	1.38±0.11	1.39±0.09
Slaughter	Female	10.9±0.76	11.7±0.63	11.5±0.27	11.5±0.64
	Male	11.4±0.63	11.7±0.59	12.1±0.51	12.0±0.75

Values are mean±S.E.M. There were no significant treatment effects or sex differences.

Results

There was no effect of pre-natal treatment on gestation length, litter size, piglet weight at birth or the within-litter variability in piglet birth weight (Table 1). There was no effect of the pre- or post-natal treatment on weight at slaughter in male or female pigs (Table 2).

Female pigs

The number of each category of ovarian follicle observed is shown in Table 3. No antral follicles were present. Primordial follicles were the most prevalent follicle type. Female pigs born to sows that were stressed during pregnancy had fewer primordial follicles than those born to control sows (Table 3). Plasma oestradiol (E_2) concentrations were lower in female pigs that had their tails amputated soon after birth (Table 3). There were no significant pre-natal stress×post-natal pain interactions on any of the traits measured in female pigs.

Male pigs

Testes collected from pigs that had their tails amputated soon after birth were lighter than those collected from intact pigs ($P=0.01$; Table 4). Male pigs born to stressed sows had lower plasma testosterone and E_2 concentrations. E_2 concentrations were also lower in male pigs that had their tails amputated (Table 4). There was a significant interaction between the effects of pre-natal stress and post-natal pain on testicular expression of 17α-OH, such that amputation increased expression in pigs born to control sows, but reduced expression in animals born to stressed sows (Table 4).

A representative stained image of a testis section is shown in Fig. 1. There were no effects of pre-natal stress or post-natal pain the number of germ cells, seminiferous tubules, Leydig cells or Sertoli cells, when expressed either per total area of testis tissue (Table 5) or as a percentage of the total cells. However, the number and percentage of Leydig cells positively stained for Ki-67

Table 3 Effect of pre-natal stress and post-natal pain on ovarian follicle development and plasma oestradiol (E_2) concentrations in female pigs.

	Control		Stressed			*P*	
	Intact	Amputated	Intact	Amputated	S.E.D.	Pre-natal stress	Amputation
Plasma E_2 (pg/ml)	7.05	4.28	6.72	5.21	0.86	NS	0.03
Primordial follicles (no./μm^2)[a]	−3.825	−4.105	−4.282	−4.342	0.15	0.027	NS
Transitory follicles (no./$\mu m^2 \times 10^{-6}$)	34.8	29.2	39.3	31.8	6.30	NS	NS
Primary follicles (no./$\mu m^2 \times 10^{-6}$)	8.402	12.288	12.134	9.144	3.65	NS	NS
Small preantral follicles (no./$\mu m^2 \times 10^{-6}$)	8.135	6.855	4.746	6.886	1.69	NS	NS
Large preantral follicles (no./$\mu m^2 \times 10^{-6}$)	1.432	1.967	2.807	1.534	1.19	NS	NS
Germ cell nests (no./$\mu m^2 \times 10^{-6}$)	3.187	0.431	2.726	0.499	1.81	NS	NS ($P=0.069$)

[a]Data are log transformed. NS, not significant. S.E.D., standard error of the difference.

was lower in pigs that had their tails amputated. There were no significant effects of the interaction between pre-natal stress and post-natal pain on any measure of testis cell type.

Discussion

This study has shown that pre- and post-natal stresses associated with routine animal husbandry practices alter reproductive development of male and female pigs. In particular, circulating concentrations of testosterone and E_2 were lower in males born to stressed sows, while tail amputation soon after birth reduced E_2 concentrations in male and female pigs, testis weight and the percentage of proliferating Leydig cells. This is believed to be the first report showing that procedures conducted during the first few days of life affect reproductive development.

The results from this study suggest that, as with the rat (Ward & Weisz 1984), testicular, but not ovarian, steroidogenesis is affected by pre-natal stress. In male pigs E_2 is synthesised in the Leydig cells of the foetal and post-natal testis (Wagner & Claus 2008) and, unlike many other species, male pigs often have higher E_2 concentrations than females. In boars, E_2 acts with androgens to regulate sexual behaviour, anabolic metabolism and accessory gland function. The concentrations of E_2 in male pigs observed in this study are similar to those previously reported in young boars of the same age (Wagner & Claus 2008).

The mechanisms by which pre-natal stress reduces post-natal testosterone and E_2 concentrations in male pigs are not known, particularly given the absence of pre-natal treatment effects on Leydig cell numbers. Previous studies using the same experimental paradigm have shown that mixed sows have elevated cortisol concentrations for up to 2 days after mixing (Jarvis *et al.* 2006, Rutherford *et al.* 2009); however, it is not known how long such elevated concentrations would persist. Pregnant rats that were stressed by restraint and bright lighting during the last 11 days of gestation had reduced placental expression of 11β-hydroxysteroid dehydrogenase (Mairesse *et al.* 2007), an enzyme that protects the foetus from high maternal cortisol concentrations. Based on these findings, it is reasonable to assume that foetuses carried by stressed sows would have elevated circulating cortisol concentrations which would provide increased negative feedback to the foetal hypothalamus and anterior pituitary, leading to a re-setting of the foetal HPA axis. It is possible that the stress-induced attenuation of the foetal HPA axis proposed by Mairesse *et al.* (2007) could also affect the foetal hypothalamic–pituitary–gonadal axis. In support of this, male foetuses carried by stressed pregnant rats failed to show the surge of testosterone normally seen on days 18 and 19 (Ward & Weisz 1984) and had lighter testes on day 21 of foetal life (Mairesse *et al.* 2007), suggesting a stress-induced depression in early testicular steroidogenesis. The sex difference in the steroidogenic response to pre-natal stress observed in this study could reflect the fact that foetal pig testes (Colenbrander *et al.* 1979), but not ovaries (Colenbrander *et al.* 1983) require continued pituitary support throughout pregnancy and would therefore be more susceptible to changes in hypothalamic–pituitary activity. In addition, the timing of

Table 4 Effect of pre-natal stress and post-natal pain on testis weight and plasma testosterone and oestradiol (E_2) concentrations in male pigs.

	Control		Stressed			*P*		
	Intact	Amputated	Intact	Amputated	S.E.D.	Stress	Amputation	Interaction
Combined testes weight (g)	12.8	11.9	11.8	9.93	0.61	NS	0.045	NS
Combined testes weight as % body weight	0.107	0.098	0.097	0.084	0.003	NS	0.01	NS
Testosterone concentrations (ng/ml)	1.063	0.650	0.577	0.507	0.226	0.014	NS	NS
E_2 concentrations (pg/ml)	45.8	31.6	25.4	20.4	8.57	0.015	0.021	NS
17α-Hydroxylase mRNA expression[a]	0.674	0.617	0.607	0.682	0.05	NS	NS	0.036

[a]Data are square-root transformed. NS, not significant. S.E.D., standard error of the difference.

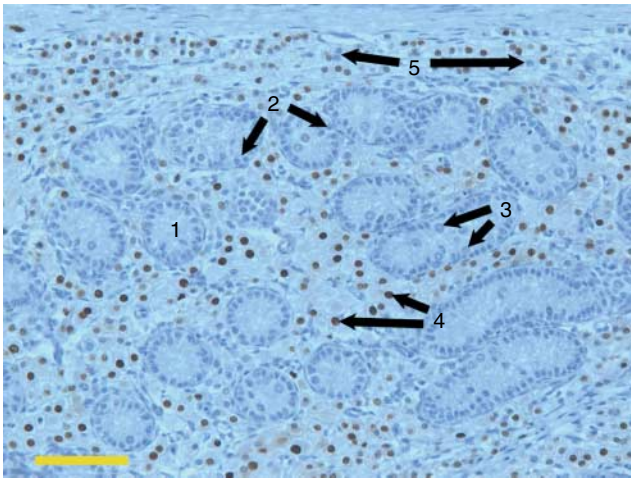

Figure 1 Representative section of day 40 pig testis showing cell types counted and Ki-67 immunostaining. Stained structures indicated by arrows are: seminiferous tubules (1), Sertoli cells (2), Germ cells (3), Leydig cells stained positive for Ki-67 (4), and Leydig cells stained negative for Ki-67 (5). Scale bar = 200 μm.

pre-natal stress coincides with waves of Leydig cell differentiation in male foetuses, but precedes the formation of primary and secondary ovarian follicles (Oxender *et al*. 1979).

It is possible that the differences in plasma E_2 and testosterone concentrations observed reflect differences in adrenal, rather than gonadal, steroid production. In what appears to be the only published study describing adrenal steroid secretion in young pigs, Holzbauer & Newport (1969) suggested that androgens and progesterone-related compounds could account for up to 30% of total adrenal steroid secretion in 8- to 12-week-old female pigs. These authors presented some evidence that adrenal gland secretion of pregnenolone and 11β-OH-androstenedione increased under stressful conditions, suggesting that altered adrenal production of E_2 or testosterone is unlikely to explain the lower concentrations of these steroids observed in this study. Furthermore, the observation that daughters of stressed sows have higher cortisol concentrations when measured on day 67 of life (Jarvis *et al*. 2006) would not suggest suppressed adrenal steroidogenesis is this group.

Other potential mechanisms should be considered. For example, observations that administration of naltrexone to stressed pregnant rats blocks the feminising effects of pre-natal stress on male offspring (Keshet & Weinstock 1995, Reznikov *et al*. 2005), led to the proposal that the opioid system may be involved in mediating the effects of stress on the reproductive axis.

An intriguing finding from this study is that, regardless of pre-natal treatment, husbandry procedures conducted soon after birth affected reproductive development in both sexes. The observation that tail-amputated male pigs had fewer proliferating Leydig cells, smaller testes and lower E_2 concentrations suggests that testicular growth and development were compromised. As both the amputated and intact groups were handled in the same way and were of equivalent weight at slaughter, the results do not appear to be a consequence of the handling stress described by Weaver *et al*. (2000) or of altered feed intake. However, work by Klemcke *et al*. (1995) showing that neonatal (12 days of age) boars had increased ACTH and cortisol responses on the day after being restrained for 1 min highlights the extreme sensitivity of neonatal pigs to a modest stressor. Assessment of nociceptive functioning in siblings of the study animals used here (Sandercock *et al*. 2011) did not find any evidence of alterations as a consequence of tail docking, so the changes to reproductive function cannot be attributed to ongoing chronic pain, or more general alterations in nociceptive threshold.

In the light of recent work showing that pre-natal stress heightens piglet response to painful procedures (Rutherford *et al*. 2009), we hypothesised that reproductive consequences of tail amputation may also be influenced by whether the pig had been exposed to pre-natal stress. The only variable measured where there was a significant pre- by post-natal treatment interaction was in the testis expression of 17α-OH, such that amputation increased expression in pigs born to control sows, but reduced expression in animals born to stressed sows. This enzyme, which catalyses the production of precursors for glucocorticoid, oestrogen and androgen synthesis is involved in sexual development during foetal life and at puberty. Increased exposure to oestrogen during mid to

Table 5 Effect of pre-natal stress and post-natal pain on the number of testis cell types and the proliferative activity of Leydig cells in male pigs.

	Control		Stressed			*P*	
	Intact	Amputated	Intact	Amputated	S.E.D.	Pre-natal stress	Amputation
Germ cells/mm^2[a]	0.244	0.246	0.273	0.221	0.031	NS	NS
Seminiferous tubules/mm^2	0.112	0.127	0.125	0.128	0.011	NS	NS
Sertoli cells/mm^2	4.53	5.01	4.84	4.90	0.48	NS	NS
Leydig cells/mm^2	2.728	2.545	3.112	3.317	0.34	NS 0.085	NS
Seminiferous tubules as proportion of total area[b]	0.328	0.375	0.350	0.3410	0.027	NS	NS
% Ki-67 positive Leydig cells[b]	2.133	2.031	2.114	1.917	0.114	NS	0.036

[a]Data are square root transformed. [b]Data are log transformed. NS, not significant. S.E.D., standard error of the difference.

late pregnancy in the rat increased foetal Leydig expression of 17α-OH (Majdic *et al.* 1996), indicating that external stressors can affect foetal steroid synthesis.

Given that the results described in this study were obtained from animals at a single pre-pubertal time point, it is not possible to say whether the treatment effects observed would be likely to persist into adult life or whether the lower hormone concentrations and cell numbers reflect developmental delay. Recent data from studies of reproductive aging (menopause) in women indicate that reproductive traits in pre-pubertal females can predict later reproductive capability. Menopause occurs when primordial ovarian follicles are depleted, despite the continued presence of a pool of later developing follicles (Appt *et al.* 2010). The number of primordial follicles between birth and puberty determines not only the total ovarian reserve, but also the rate at which primordial follicles are recruited into the pool of growing follicles. The number of follicles recruited is inversely related to the number of non-growing (largely primordial) follicles (Hansen *et al.* 2008) such that females with fewer primordial follicles before puberty would be expected to reach reproductive senescence earlier. The relationship between the number of primordial ovarian follicles and reproductive aging in livestock species has not been studied. Given that lifetime reproductive performance of breeding females and the testosterone-driven growth potential of intact males are important traits for the pig industry, the relationships between early life experiences and commercially relevant reproductive traits warrant further study.

Materials and Methods

Experimental animals

In this study, 64 39- to 43-day-old pigs were used. These pigs were born to 16 Large White–Landrace primiparous sows that had either been stressed (S, $n=8$ sows) by mixing with unfamiliar older sows for two 1-week periods during mid-pregnancy, or not (control, C, $n=8$ sows). Before insemination and through to mid-pregnancy sows remained in socially stable social groups of five or six animals. Two or three sows from each group were pre-allocated to the social stress treatment. The remaining sows in each group were controls. Allocation to the S group was made on the basis of a dominance test conducted in the home pen before the mixing period, and was balanced to ensure equal proportions of dominant, intermediate and subordinate animals in S and C groups. Oestrous cycles were not synchronised in study animals so on the first mix day S animals were on average on day 47 of gestation (range: 44–50). On the first day of mixing, sows allocated to the stress group were moved to a different pen containing three larger multiparous sows, where they were kept for 1 week, before being returned to their home pen. After 1 week in the home pen S sows were mixed again, with a different batch of sows, for one further week. Over this whole period control sows were left undisturbed in their home pen. Following the second mix period, all sows were left undisturbed in their original social group until being moved to standard farrowing crates 5 days before expected parturition around 114 days after insemination. Litter size and individual piglet birth weight was recorded.

On day 3 after birth half the female piglets and half the male piglets in each litter had their tails amputated (A) using a pair of surgical cutters. The remaining piglets were sham operated but remained intact (I). Piglets were weaned at around 28 days (mean $\pm$ S.D. $=28\pm2.7$; no difference between S and C groups) and housed in their litter groups in straw-bedded pens. On days 39–43 of age four pigs from each litter, representing each gender and post-natal treatment combination (male I and A, female I and A) were weighed and sedated with an i.m. injection of 2 mg/kg azaperone (Stresnil; Janssen Animal Health, High Wycombe, Buckinghamshire, UK) mixed with 5 mg/kg ketamine (Vetalar; Boehringer Ingelheim, Bracknell, Berkshire, UK). Once sedated, each pig received an intra-cardiac injection of 15 ml of sodium pentobarbital (Euthatal; Merial Animal Health, Harlow, Essex, UK) to ensure rapid death. Immediately after death, the pig was exsanguinated and ~10 ml of whole blood collected. Both gonads were removed from each pig and the testes weighed. One testis from each male pig was cut into four roughly equivalent transverse sections, and one ovary from each female was bisected longitudinally. These tissue pieces were placed in Bouin's solution (Sigma–Aldrich Ltd) at room temperature. Similarly dissected pieces of the remaining gonad were snap frozen in liquid nitrogen and stored at −80 °C.

All experimental procedures were approved by the Scottish Agricultural College Animal Ethics Committee in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Histology of ovary and testes

After 24 h the Bouin's solution was replaced with 70% ethanol and the fixed samples stored at room temperature to await further processing. Samples were transferred to histological cassettes, dehydrated and embedded in paraffin. Sections (5 μm) were cut using a microtome and placed on a microscope slide.

Two slides from each ovary were stained with haematoxylin and eosin. Bright field pictures of one section on each slide were taken at four pre-determined positions at magnifications of 10× and 20× on a Leica DMRB microscope fitted with a digital camera and the images analysed using ImageJ Software (http://rsb.info.nih.gov/ij/). The developmental stage of ovarian follicles with visible oocyte nuclei was classified as described by Lundy *et al.* (1999). This classification is based on the number and structure of granulosa cells surrounding the follicle, ranging from primordial follicles with one layer of flattened granulosa cells to small antral follicles with more than five complete layers of cuboidal granulosa cells with a fully formed antrum. Clusters of cells that were not individually surrounded by granulosa cells were classified as egg cell nests. The number of ovarian follicles in each category was expressed relative to the area of the field of view.

The nuclear protein Ki-67 was detected in testis sections by immunohistochemistry as a marker of cell proliferation (Bullwinkel *et al.* 2006). Following dewaxing and antigen

retrieval using 0.01 M sodium citrate, endogenous peroxidase activity, endogenous protein-associated biotin and non-specific antibody binding sites were blocked by sequentially incubating slides with 3% H_2O_2, Avidin D and biotin solution (Vector Laboratories, Peterborough, UK) and normal horse serum in 5% BSA. Two slides from each gonad were incubated with mouse MAB to Ki-67 (Ab17106; Abcam; 1:25) and two with mouse IgG (2 μg/ml) as the negative control. The slides were incubated overnight at +4 °C in a humidified chamber followed by 60 min at room temperature with a biotinylated horse anti-mouse secondary antibody (Vector Laboratories). An avidin–biotin HRP complex (Vector Laboratories), which binds to the biotin-labelled antibody, was added and detected using the peroxidase substrate, diaminobenzidene (Vector Laboratories). Testes slides were counterstained with haematoxylin (VWR, Lutterworth, UK). Images from four pre-determined positions from each slide were captured at 10× magnification using a Nikon Optishot 2 microscope and analysed by ImageJ. The area of the field of view and the area of seminiferous tubules were determined. Numbers of germ cells, seminiferous tubules, Sertoli cells and Leydig cells were counted. The number of Ki-67 positive and negative Leydig cells was counted. From these data the number of each testis cell type per square millimeter and the percentage of the total cells represented by each cell type were calculated, as were the number of Sertoli cells/seminiferous tubule.

E_2 and testosterone assays

Blood samples were centrifuged at 1500 ***g*** and 4 °C for 10 min and the plasma decanted and frozen at −20 °C to await analysis. Testosterone and total unconjugated E_2 concentrations were determined by RIA using 'coat-a-count' kits (Siemens Healthcare Diagnostics, Deerfield, IL, USA) as previously described in pig plasma (Andersson *et al.* 1998, Landerdijk *et al.* 2009). Serially diluted pig plasma samples gave results parallel to the standard curve. In addition, in-house standards of known steroid concentration assayed using the coat-a-count kit gave the expected concentrations. All samples from female pigs were analysed in one E_2 assay and those from male pigs in another. E_2 was extracted from plasma using diethyl ether. The mean extraction efficiency was 81.7%. Individual samples were corrected for extraction efficiency. The minimum detectable dose was 3 pg/ml and the mean inter-assay coefficient of variation using three quality control samples was 4.35%. Testosterone concentrations in plasma samples from male animals were determined directly in a single RIA. The minimum detectable dose was 0.08 ng/ml.

Total RNA extraction and cDNA synthesis

Approximately, 50 mg of frozen testis tissue was homogenised in 1 ml RNA-Bee (AMS Biotechnology, Abingdon, Oxford, UK), 0.2 ml chloroform was added and the sample centrifuged at 12 000 ***g*** for 15 min at 4 °C. The aqueous layer was removed, an equal volume of 70% ethanol added and the sample transferred to an RNeasy Mini spin column (Qiagen) and the column processed according to the manufacturer's instructions. Extracted RNA was stored at −80 °C. The concentration of RNA was determined spectrophotometrically (NanoDrop Thermo-Scientific, Wilmington, DE, USA). The quality of the RNA was determined on an Agilent Technologies Bioanalyser (Wokingham, Berkshire, UK). The average RNA integrity number was 9.6 (range: 8.9–10).

cDNA was prepared from duplicate samples containing 1 μg of isolated RNA by RT using random primers (Promega) and Superscript III (Invitrogen) and stored at −20 °C. Controls containing no DNA transcript or no Superscript III were used to test for reagent contamination and genomic DNA contamination.

Relative expression of 17α-OH in testis

The relative expression of *17α-OH* transcripts in testis tissue was measured by qPCR using Platinum SYBR Green SuperMix UTD (Invitrogen) in accordance with the MIQE guidelines (Bustin *et al.* 2009). Following screening of the nine genes identified by Nygard *et al.* (2007) as suitable reference genes for expression studies in pig tissues, geNorm was used to select the three most stable genes: *HMBS, SDHA* and *YWHA2*. The primer sequences of the four genes used are shown in Table 6.

Duplicate samples of both cDNA samples prepared from each testis were analysed. Serial dilutions of pooled cDNA ranging from 1:2 to 1:64 in nuclease-free water were used as standards. Duplicate cDNA samples from one of the two cDNA preparations/testis, standards and controls containing no cDNA

Table 6 Porcine-specific primer sequences for qPCR.

Primer names	**Sequence** (5′–3′)	**Amplicon size** (bp)	**Tm** (°C)	**Accession numbers**
17α-OH		202		M63507
Forward	CTGTGGGCAAGGAAATTTTG			
Reverse	ACTTTCTGCGTTCGTCTTGG			
HMBS2		83	58	DQ845174
Forward	AGGATGGGCAACTCTACCTG			
Reverse	GATGGTGGCCTGCATAGTCT			
SDHA		141	58	DQ845177
Forward	CTACAAGGGGCAGGTTCTGA			
Reverse	CTACAAGGGGCAGGTTCTGA			
YWHAZ		203	60	DQ845179
Forward	TGATGATAAGAAAGGGATTGTGG			
Reverse	GTTCAGCAATGGCTTCATCA			

were included on each plate. Each qRT-PCR well contained 12.5 μl platinum SYBR Green qPCR supermix (Invitrogen), 0.1 μl ROX reference dye, 0.2 μl (5 μM) each of forward and reverse primer and 3.4 μl nuclease-free water. Normalisation factors for the three reference genes were calculated using the geNorm applet for Microsoft Excel as described by Vandesompele *et al.* (2002). The mean slope and intercept for the 17α-OH calibration curves were −3.363 and 15.47 threshold cycle (C_t) (dRn) respectively. The PCR efficiency was 98.3%.

Statistical analysis

Testes weights were expressed as a percentage of pig weight at slaughter.

The effects of the pre- and post-natal treatment and their interaction were analysed by two-way ANOVA (Genstat Version 13.1 for Windows; VSN International Ltd, Oxford, UK). For any specific comparison $n=8$, but litter was always the unit of measurement. The litter term was blocked to allow comparisons between intact and amputated same-sex siblings. Age at slaughter was fitted as a covariate. To overcome scale effects some data were log (number of primordial ovarian follicles, % Ki-67 positively stained Leydig cells and the area of seminiferous tubules) or square root (testes expression of 17α-OH and number of germ cells in the testes) transformed before analysis. Differences were considered significant when $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Andersson H, Rydhmer L, Lundstrom K, Wallgren M, Andersson K & Forsberg M 1998 Influence of artificial light regimens on sexual maturation and boar taint in entire male pigs. *Animal Reproduction Science* **51** 31–43. (doi:10.1016/S0378-4320(98)00054-2)

Appt SE, Clarkson TB, Hoyer PB, Kock ND, Goode AK, May MC, Persyn JT, Vail NK, Ethun KF, Chen H *et al.* 2010 Experimental reduction of reduced ovarian reserve in a nonhuman primate model (*Macca fascicularis*). *Comparative Medicine* **60** 380–388.

Ashworth CJ, McEvoy TG, Rooke JA & Robinson JJ 2005 Nutritional programming of physiological systems throughout development. *Trends in Developmental Biology* **1** 117–129.

Bielli A, Perez R, Pedrana G, Milton JTB, Lopez A, Blackberry MA, Duncombe G, Rodreguez-Martinez H & Martin GB 2002 Low maternal nutrition during pregnancy reduces the number of Sertoli cells in the newborn lamb. *Reproduction, Fertility, and Development* **14** 333–337. (doi:10.1071/RD02046)

Bullwinkel J, Baron-Luhr B, Ludemann A, Wohlenberg C, Gerdes J & Scholzen T 2006 Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *Journal of Cellular Physiology* **206** 624–635. (doi:10.1002/jcp.20494)

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al.* 2009 The MIQE guidelines: minimum information for publication of real-time PCR experiments. *Clinical Chemistry* **55** 611–622. (doi:10.1373/clinchem.2008.112797)

Colenbrander B, Van Rossum-Kok CMJE, Van Staaten HWM & Wensing CJG 1979 The effect of fetal decapitation on the testis and other endocrine organs in the pig. *Biology of Reproduction* **20** 198–204. (doi:10.1095/biolreprod20.2.198)

Colenbrander B, Van Rossum-Kok CMJE, Oxender WD & Wensing CJ 1983 Ovarian development in control and decapitated pig fetuses. *Biology of Reproduction* **20** 198–204. (doi:10.1095/biolreprod20.2.198)

Gardner DS, Lea RG & Sinclair KD 2008 Developmental programming of reproduction and fertility: what is the evidence? *Animal* **2** 1128–1134. (doi:10.1017/S1751731108002607)

Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR & Klein NA 2008 A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Human Reproduction* **23** 699–708. (doi:10.1093/humrep/dem408)

Harker LE, Duncan WC, Rutherford K, Rooke JA, Lawrence AB & Ashworth CJ 2008 Effect of early life stressors on reproductive capability in pigs. *Proceedings of the Annual Meeting of the Society of Reproduction and Fertility,* Edinburgh, UK. Abstract P63, pp 75.

Holzbauer M & Newport HM 1969 Adrenal secretion rates and adrenal tissue concentrations of pregnenolone, progesterone, 11βOH-androstenedione and some other steroids in young pigs and dogs. *Journal of Physiology* **200** 821–848.

Jarvis S, Moinard C, Robson SK, Baxter E, Ormandy E, Douglas AJ, Seckl JR, Russell JA & Lawrence AB 2006 Programming the offspring by prenatal social stress: neuroendocrine activity and behaviour. *Hormones and Behavior* **49** 68–80. (doi:10.1016/j.yhbeh.2005.05.004)

Kapoor A & Matthews SG 2005 Short periods of prenatal stress affect growth, behaviour and hypothalamo-pituitary–adrenal axis activity in male guinea pig offspring. *Journal of Physiology* **566** 967–977. (doi:10.1113/jphysiol.2005.090191)

Kattesh HG, Kornegay ET, Gwazdauskas FC, Knight JW & Thomas HR 1979 Peripheral plasma testosterone concentration and sexual behaviour in young prenatally stressed boars. *Theriogenology* **12** 289–305. (doi:10.1016/S0093-691X(79)80009-6)

Keshet GI & Weinstock M 1995 Maternal naltrexone prevents morphological and behavioral alterations induced in rats by prenatal stress. *Pharmacology, Biochemistry, and Behavior* **50** 413–419. (doi:10.1016/0091-3057(94)00289-U)

Klemcke HG, Brown-Borg HM & Borg KE 1995 Functioning of the porcine pituitary–adrenocortical axis during neonatal development. *Biology of the Neonate* **67** 274–286. (doi:10.1159/000244175)

Landerdijk P, Dieleman SJ, van Dooremalen C, Foxcroft GR, Gerritsen R, Hazeleger W, Soede NM & Kemp B 2009 LH and FSH secretion, follicle development and oestradiol in sows ovulating or failing to ovulate in an intermittent suckling regimen. *Reproduction, Fertility, and Development* **21** 313–322. (doi:10.1071/RD06172)

Lay DC, Kattesh HG, Cunnick JE, Daniels MJ, McMunn KA, Toscano MJ & Roberts MP 2008 Prenatal stress effects on pig development and response to weaning. *Journal of Animal Science* **86** 1316–1324. (doi:10.2527/jas.2007-0812)

Lundy T, Smith P, O'Connell A, Hudson NL & McNatty KP 1999 Populations of granulosa cells in small follicles of the sheep ovary. *Journal of Reproduction and Fertility* **115** 251–262. (doi:10.1530/jrf.0.1150251)

Mairesse J, Lesage J, Breton C, Breant B, Hahn T, Darnaudery M, Dickson SL, Seckl J, Blondeau B, Vieau D *et al.* 2007 Maternal

stress alters endocrine function of the feto-placental unit in rats. *American Journal of Physiology. Endocrinology and Metabolism* **292** E1526–E1533. (doi:10.1152/ajpendo.00574.2006)

Majdic G, Sharpe RM, O'Shaughnessey PJ & Saunders PT 1996 Expression of cytochrome P450 17alpha-hydroxylase/C17-20 lysase in the fetal rat testis is reduced my maternal exposure to exogenous estrogens. *Endocrinology* **137** 1063–1070. (doi:10.1210/en.137.3.1063)

Marchlewska-Koj A, Kruczek M, Kapusta J & Pochron E 2003 Prenatal stress affects the rate of sexual maturation and attractiveness in bank voles. *Physiology & Behavior* **79** 305–310. (doi:10.1016/S0031-9384(03)00099-4)

Nygard AB, Jorgensen CB, Cirera S & Fredholm M 2007 Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Molecular Biology* **8** 67. (doi:10.1186/1471-2199-8-67)

Oxender WD, Colenbrander B, van deWiel DFM & Wensing CJG 1979 Ovarian development in fetal and pubertal pigs. *Biology of Reproduction* **21** 715–721. (doi:10.1095/biolreprod21.3.715)

Rae MT, Kyle CE, Miller DW, Hammond AJ, Brooks AN & Rhind SM 2002 The effects of undernutrition, *in utero,* on reproductive function in adult male and female sheep. *Animal Reproduction Science* **72** 63–71. (doi:10.1016/S0378-4320(02)00068-4)

Reznikov AG, Nosenko ND & Tarasenko LV 2005 Opiods are responsible for neurochemical feminisation of the brain in prenatally stressed rats. *Neuroendocrinology Letters* **26** 35–38.

Rhind SM 2004 Effects of maternal nutrition on fetal and neonatal reproductive development and function. *Animal Reproduction Science* **122** 205–214.

Rutherford K, Robson S, Donald R, Jarvis S, Sandercock D, Scott M, Nolan A & Lawrence AB 2009 Prenatal stress amplifies the immediate behavioural responses to acute pain in pigs. *Biology Letters* **5** 452–454. (doi:10.1098/rsbl.2009.0175)

Sandercock DA, Gibson IF, Rutherford KMD, Donald RD, Lawrence AB, Brash HM, Scott EM & Nolan AM 2011 The impact of prenatal stress on basal nociception and evoked responses to tail-docking and inflammatory challenge in juvenile pigs. *Physiology & Behavior* **104** 728–737. (doi:10.1016/j.physbeh.2011.07.018)

Sutherland MA, Bryer PJ, Krebs N & McGlone JJ 2008 Tail docking in pigs: acute physiological and behavioural responses. *Animal* **2** 292–297. (doi:10.1017/S1751731107001450)

Vandesompele J, de Preter K, Pattyn F, Poppe B, van Roy N, de Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (doi:10.1186/gb-2002-3-7-research0034)

Wagner A & Claus R 2008 Aromatase and 11β-hydroxysteroid dehydrogenase 2 localisation in the testes of pigs from birth to puberty linked to changes of hormone pattern and testicular morphology. *Reproduction, Fertility, and Development* **20** 505–512. (doi:10.1071/RD07136)

Ward IL & Weisz J 1984 Differential effects of maternal stress on circulating levels of corticosterone, progesterone, and testosterone in male and female rat fetuses and their mothers. *Endocrinology* **114** 1635–1644. (doi:10.1210/endo-114-5-1635)

Weaver SA, Aherne FX, Meaney MJ, Schaefer AL & Dixon WT 2000 Neonatal handling permanently alters hypothalamic–pituitary–adrenal axis function, behaviour and body weight in boars. *Journal of Endocrinology* **164** 349–359. (doi:10.1677/joe.0.1640349)

Williams MT, Hennessy MB & Davis HN 1998 Stress during pregnancy alters rat offspring morphology and ultrasonic vocalisations. *Physiology & Behavior* **63** 337–343. (doi:10.1016/S0031-9384(97)00428-9)

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